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Application of solid-phase microextraction to the quantitative analysis of 1,8-cineole in blood and expired air in a *Eucalyptus* herbivore, the brushtail possum (*Trichosurus vulpecula*)

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Abstract

We have developed two solid-phase microextraction (SPME) methods, coupled with gas chromatography, for quantitatively analysing the major *Eucalyptus* leaf terpene, 1,8-cineole, in both expired air and blood from the common brushtail possum (*Trichosurus vulpecula*). In-line SPME sampling (5 min at 20 °C room temperature) of excurrent air from an expiratory chamber containing a possum dosed orally with 1,8-cineole (50 mg/kg) allowed real-time semi-quantitative measurements reflecting 1,8-cineole blood concentrations. Headspace SPME using 50 μl whole blood collected from possums dosed orally with 1,8-cineole (30 mg/kg) resulted in excellent sensitivity (quantitation limit 1 ng/ml) and reproducibility. Blood concentrations ranged between 1 and 1380 ng/ml. Calibration curves were prepared for two concentration ranges (0.05–10 and 10–400 ng/50 μl) for the analysis of blood concentrations. Both calibration curves were linear (r^2=0.999 and 0.994, respectively) and the equations for the two concentration ranges were consistent.
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Keywords: *Trichosurus vulpecula*; Breath analysis; 1,8-Cineole

1. Introduction

Over the last few years the versatility of solid-phase microextraction (SPME) has proven useful in the detection of a number of drugs and other xenobiotics in blood, plasma and other biological matrices [1–4]. Combined with gas chromatography–mass spectrometry (GC–MS), SPME has also found many applications in the sciences of flavour and fragrance with its ability to dissect the complex aromas of terpenes and other volatile compounds [5–8]. In some instances the method has been developed further to allow quantitation of analytes [9,10]. In this study we report the development of two methods for quantifying a dietary terpene, 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane), in expired air and whole blood from a herbivore, the common brushtail possum (*Trichosurus vulpecula*), for the purpose of investigating pharmacokinetic parameters of 1,8-cineole.

The brushtail possum is a small to medium

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(approximately 2–5 kg) marsupial found throughout most areas of Australia. It relies on *Eucalyptus* foliage to variable degrees for its diet. *Eucalyptus* leaves contain substantial amounts of volatile terpenes that constitute an important component of their chemical defence system, yet the brushtail possum can ingest quantities of terpenes that would be toxic to other animal species, including humans. Measuring the pharmacokinetics of terpenes in the possum is necessary for understanding how this species detoxifies plant toxins as well as how blood levels may affect foraging behaviour. For this purpose it was necessary to develop appropriate assays.

The use of breath analysis as an indirect measure of blood concentrations of volatile compounds is well established [11]. A number of human studies have detected unchanged monoterpenes in expired air after inhalation exposure [12–14]. SPME has been used in the quantitative determination of small volatile organic compounds in human expired breath [15,16]. We successfully combined the two methodologies to analyse expired air for 1,8-cineole using SPME. Unlike previous protocols for measuring expired air, we developed in-line sampling of excurrent air from an expiratory chamber. This enables determination of the rates of absorption and elimination of an oral dose of 1,8-cineole. This method had the added benefit of being non-invasive, a desirable requirement when studying wild-caught animals.

A direct assay of 1,8-cineole in whole blood was also developed: this had greater sensitivity and was readily calibrated to give concentrations in absolute units. The available blood volume per sample was limited because of the relatively small blood volume (57 ml/kg [17], or approximately 230 ml for a 4 kg possum), the cumulative effect of multiple sampling (24 samples per experiment), repeated experiments (three per possum) and extra losses from the disposal of the catheter dead volume. Generally the volume of each sample collected was 300 μl which was divided between two assays (blood was also assayed for 1,8-cineole metabolites by liquid extraction). We used a blood volume of 50 μl for the determination of 1,8-cineole in whole blood using headspace (HS) SPME.

This work applies the SPME method, coupled with GC, to a novel application of sampling and quantification of volatile dietary toxins. The method is simple, rapid and requires minimal sample preparation, yet yields high sensitivity and excellent reproducibility.

2. Experimental

2.1. Chemicals and materials

1,8-Cineole was supplied by Sigma (Castle Hill, Australia) and (−)-linalool (3,7-dimethyl-3-hydroxy-1,6-octadiene) was from Robertet Natural Concentrates, supplied by Shiono (Japan). Methyl cellulose 400 was supplied by BDH (Poole, UK).

2.2. Animals

Brushtail possums were caught in wire cage traps around the Hobart area, under permit from the Parks and Wildlife Service (Department of Primary Industries, Water and Environment, Tasmania, Australia). Approval for this study was obtained from Animal Ethics and Experimentation Committee of the University of Tasmania. Possums were housed in covered, outdoor enclosures equipped with aerial branches and a nest box for each possum. They were maintained on a diet of grated silver beet, carrot, apple, lucerne chaff and sugar [18]. Possums were weighed regularly and their masses remained constant throughout their captivity.

2.3. Instrumentation

The SPME apparatus consisted of a manual reusable syringe assembly with a 100 μm polydimethylsiloxane (PDMS) fibre (Supelco, Bellefonte, PA, USA). New fibres were conditioned for at least 1 h at 250 °C in the GC injector. Although the fibre specifications recommend 100 injections per fibre, we were able to use each fibre many times more than this. The performances of fibres were monitored closely, and assay sensitivity was often maintained after an excess of 500 injections.

Gas chromatography was performed on a Varian 3300 gas chromatograph fitted with a Varian split/splitless capillary injector, flame ionisation detection (FID) system and Star Workstation (Version 5.31; Varian, Walnut Creek, CA, USA). Chromatography

of expired air samples was carried out on a 24 m HP-1 capillary column (0.32 mm I.D., coated with 0.52 μm cross linked 1% phenyl methyl silicone; Hewlett-Packard Australia Limited, Melbourne, Australia) fitted with a replaceable 10 cm HP-1 pre-column. The GC operating conditions were: splitless injector 220 °C, relay on 2.0 min, detector 300 °C, oven 90 °C for 2 min and then 20 °C/min to 170 °C, then 40 °C/min to 250 °C and held for 1 min, carrier gas He at a pressure of 7 p.s.i. (1 p.s.i. = 6894.76 Pa). The SPME fibre was desorbed in the GC injector for 2 min. Chromatography of blood samples was carried out on a 30 m Econocap SE54 capillary column (0.32 mm I.D., coated with 0.25 μm 5% phenyl–95% methylpolysiloxane; Alltech Associates, Deerfield, IL, USA). GC operating conditions were: splitless injector 220 °C, relay on 2.0 min, detector 300 °C, oven 60 °C for 2 min and then 20 °C/min to 240 °C, carrier gas He at a pressure of 9 p.s.i. and SPME fibre desorption of 2 min.

A heating block (Lab-Line, Melrose Park, IL, USA) was used to maintain the temperature of vials at 35 °C during absorption from the headspace of blood samples.

2.4. Expired air experiments

A mixture of 1,8-cineole (100 mg/ml) in methyl cellulose (2%) mucilage was prepared. Possums were dosed with 1,8-cineole 50 mg/kg intragastrically using a flexible paediatric feeding tube (8 French Gauge ×40 cm, Indoplas, Maersk Indoplas, Sydney, Australia). This dosage was chosen based on an estimated intake of 1,8-cineole from a single feeding bout. Previous experience has shown it to be well tolerated by possums [19].

Possums were quickly transferred to a purpose-made 15 l all-glass expiration chamber held securely on a wooden frame (Fig. 1). The front of the chamber was sealed with an outlet cover and a constant airflow of 5 l/min established using an electric pump (Air-Cadet Model 7530, Cole-Parmer, Vernon Hills, IL, USA). Airflow through the system was based on the recommendation that an adult possum would require a minimum of 0.5 ml of oxygen/g body mass/h, when resting at thermoneutrality (Dr. Stewart Nicol, Department of Anatomy and Physiology, University of Tasmania, personal communication). Therefore, to maintain oxygen levels around 20%, a minimum flow-rate of 4 l/min was required.

Air inlets were positioned in the rear wall and floor of the chamber, and the latter also functioned as a urine drainage port. The possums rested on a stainless steel mesh a few mm above the floor. Turbulence was created in the excurrent air by glass baffles positioned inside the tubing walls immediately downstream from the chamber, breaking the

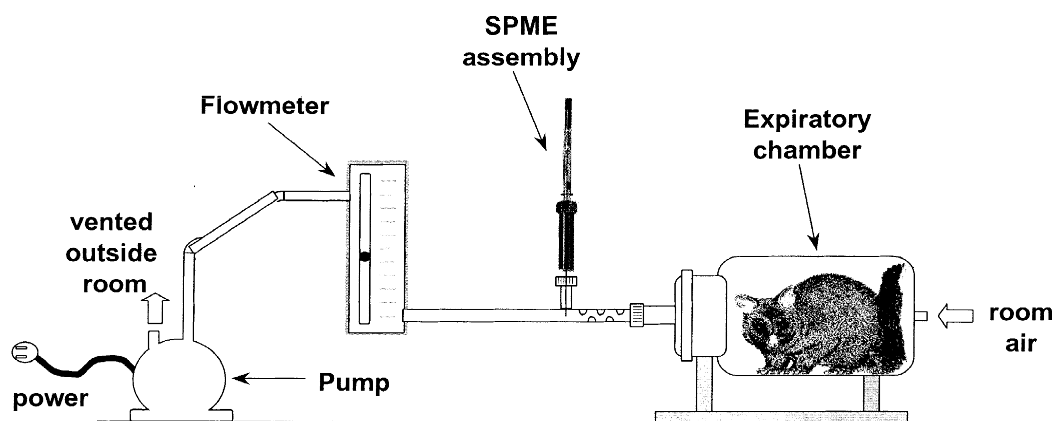

Fig. 1. Schematic of apparatus used for expired air analyses. After being dosed possums were placed in the chamber (which was darkened with a cloth cover). The chamber was sealed and connected to a pump (5 l/min) which drew air through the chamber. Turbulence was established in the excurrent air and the SPME fibre inserted into this flow. SPME absorption time was 5 min exactly.

line of sight. Excurrent air was vented outside the experimental room. The room was kept at a constant 20 °C throughout the experiment.

The SPME fibre sheath was introduced into the turbulent excurrent air via an introducing arm which was sealed with a PTFE lined silicone GC septum (Alltech Associates Australia, Baulkham Hills, Australia). The length of this side arm was such that the filament was positioned in the centre of the turbulent excurrent air when exposed. Fibre absorption time was exactly 5 min for each sampling period and samples were taken every 12–15 min for up to 3 h.

2.5. Blood analyses

Possums were dosed with 1,8-cineole 30 mg/kg intragastrically as described above. Blood (0.3 ml) was collected at t=0, 5, 10, 20, 30, 40, 50 and 60 min, 1.15, 1.30, 1.45, 2.00 h and at then at 30-min intervals until 6 h after the dose. Blood samples were placed in glass vials (12×32 mm Clear Vials, Bonnet Equipment, Australia) sealed with a PTFE lined rubber septa and screw caps. Each vial contained 100 IU of dried heparin (David Bull Labs., Mulgrave, Australia) and were chilled immediately in salted ice, before freezing at −18 °C for storage.

Prior to analysis blood was thawed to room temperature (about 20 °C), vortexed and 50 μl measured into a 17×60 mm glass screw cap vial (Packard Bioscience, Australia). Water (25 μl) and internal standard (250 ng linalool in 25 μl water) were also added to the vial using a 25-μl glass microsyringe to make a final sample volume of 100 μl. The vials were tightly capped using PTFE lined silicon septa. The septa were prepunctured immediately before analysis using a hypodermic needle to facilitate the insertion of the fibre sheath needle. The fibre sheath was inserted through the septum to a depth of 1.5 cm to position the exposed fibre in the centre of the headspace. The vial was placed into a heating block to maintain temperature at a constant 35 °C and the SPME assembly unit clamped securely. Absorption time was 12 min. No stirring was required.

After absorption, the SPME fibre unit was transferred immediately to the GC injector for desorption and GC analysis as described above.

Because of the number of samples, analyses for each experiment were performed randomly over 2 days. Fresh calibration curves were prepared each day using blood collected immediately before the administration of the 1,8-cineole.

2.5.1. Optimisation of SPME absorption conditions

The following parameters for SPME absorption were optimised for blood analyses. Three replicates were prepared for each variable by measuring 50 μl blank blood into separate vials and adding 25 μl of an aqueous stock solution of 1,8-cineole (5 ng/μl; prepared from a solution of 1,8-cineole dissolved in methanol, 5 mg/ml) and 25 μl of an aqueous stock solution of linalool (10 ng/μl; prepared from an initial solution of linalool dissolved in methanol, 5 mg/ml). The analysis was as described above.

2.5.1.1. Temperature. Sampling temperatures of 30, 35, 40 and 50 °C were tested to determine an appropriate temperature for absorption. The absorption time was 12 min.

2.5.1.2. Time. SPME sampling times of 4, 8, 12, 16 and 20 min were tested to determine an appropriate absorption period. The absorption temperature was 35 °C. GC peak areas for 1,8-cineole and linalool were plotted versus absorption time to determine the time required for stabilisation of the system under the described conditions.

2.5.1.3. Sample volume. Dilution of spiked blood resulting in 0.1, 0.5 and 1.0 ml final sample volumes was investigated. An appropriate volume of distilled water was added to make up the final volume in the 0.5 and 1.0 ml samples. Absorption was carried out at 35 °C for 12 min.

2.5.1.4. Depletion of sample. A single sample of blood spiked with 1,8-cineole and linalool was prepared and repeatedly sampled. The peak areas of 1,8-cineole and linalool were recorded and the percent of sample depleted calculated by dividing the peak area by the peak area of the previous sample.

2.5.2. Calibration curve

Four terpenes (limonene, citronellal, terpinolene and linalool) were tested for their suitability as

internal standard for 1,8-cineole using HS-SPME. Linalool was chosen as the most appropriate internal standard on the basis of retention time and peak size and shape. A stock solution of linalool in methanol (5 mg/ml) was further diluted with distilled water to produce an aqueous solution of linalool (10 ng/μl). A 25-μl volume of the aqueous linalool stock solution (250 ng) was added as the internal standard to each sample.

Because of the large range of 1,8-cineole concentrations found in blood, it was necessary to prepare two calibration curves covering different concentration ranges. Each calibration curve was prepared from six standards. Each standard was prepared from 50 μl of blank blood, 25 μl internal standard (250 ng linalool in 25 μl water) and an appropriate dilution of 1,8-cineole in 25 μl of water. 1,8-Cineole was first dissolved in methanol (5.0 mg/ml) and further diluted with water to make intermediate standards (0.01, 0.1, 0.5, 5, 10 and 20 ng/μl). The intermediate standards were diluted appropriately to achieve the final standard concentrations which were: 0.05, 0.1, 0.5, 1.0, 5 and 10 ng/50 μl blood and 10, 25, 50, 125, 250 and 400 ng/50 μl for the low and high concentration ranges, respectively. The lowest calibration standard, 0.05 ng/50 μl (1 ng/ml) was considered to be the lower limit of reliable quantitation of this assay using FID.

The calibration curves were plotted using the peak area ratio of 1,8-cineole and linalool versus 1,8-cineole concentration.

2.5.3. Repeatability

Repeatability of the assay was determined for within-day and between-day variability using a low and a high concentration of 1,8-cineole. Within-day concentrations analysed were 25 and 250 ng/50 μl of blood and for between-day variability 10 and 507 ng/50 μl were used.

Results are expressed as mean±SD.

3. Results

3.1. Expired air analyses

Fig. 2 shows a typical gas chromatogram using SPME to analyse excurrent air from the possum chamber after oral administration of 1,8-cineole (50 mg/kg). Fig. 3 shows the relative concentrations of

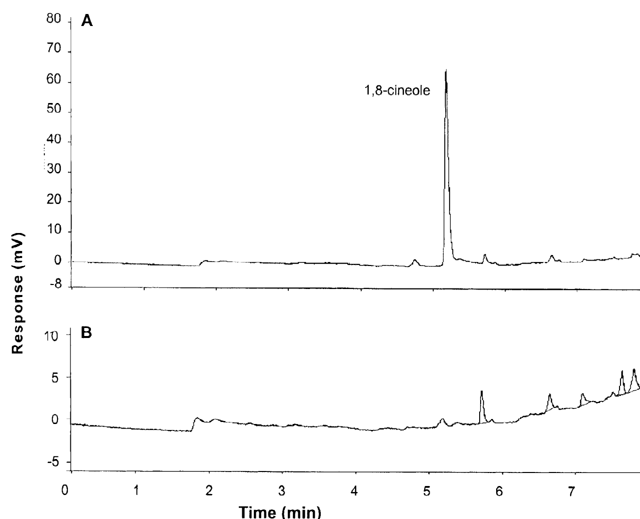

Fig. 2. Gas chromatograms (FID) of expired air from a possum collected (A) 14–19 min after a 1,8-cineole dose (50 mg/kg) and (B) prior to the administration of 1,8-cineole (control). 1,8-Cineole retention time was 5.18 min.

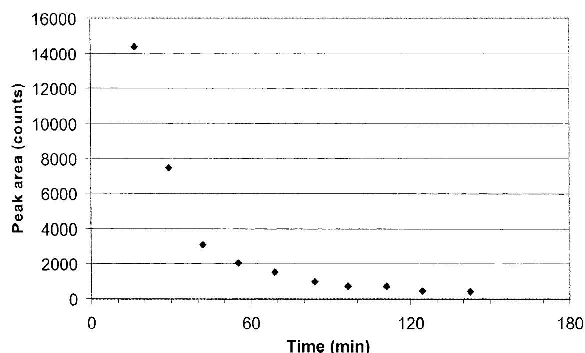

Fig. 3. Expired air 1,8-cineole time profile from a possum after an oral dose of 1,8-cineole (50 mg/kg). N.B. Concentrations are plotted at the starting time of the 5 min absorption period.

1,8-cineole in expired air for 140 min after the 1,8-cineole dose was administered.

3.2. Blood analyses

3.2.1. Optimisation of SPME absorption conditions

3.2.1.1. Temperature. The amount of 1,8-cineole absorbed onto the fibre declined while the amount of linalool increased across the temperature range of 35–50 °C (Fig. 4A). A sampling temperature of 35 °C was chosen as the optimum temperature for 1,8-cineole absorption.

3.2.1.2. Time. The effect of absorption time for 1,8-cineole and linalool is shown in Fig. 4B. The analytes had reached equilibrium with the fibre by 16 min at the optimum sampling temperature of 35 °C. We chose an absorption period of 12 min, by which time >90% of the maximum 1,8-cineole had absorbed onto the fibre.

3.2.1.3. Sample volume. Fig. 4C shows the effect of increasing the sample volume by diluting the blood with water while maintaining the above absorption temperature and time. Assay sensitivity for both analytes declined dramatically with increasing sample volume.

3.2.1.4. Sample depletion. Repeated sampling of a single vial indicates the extent of analyte depletion. 1,8-Cineole was depleted at a faster rate than the

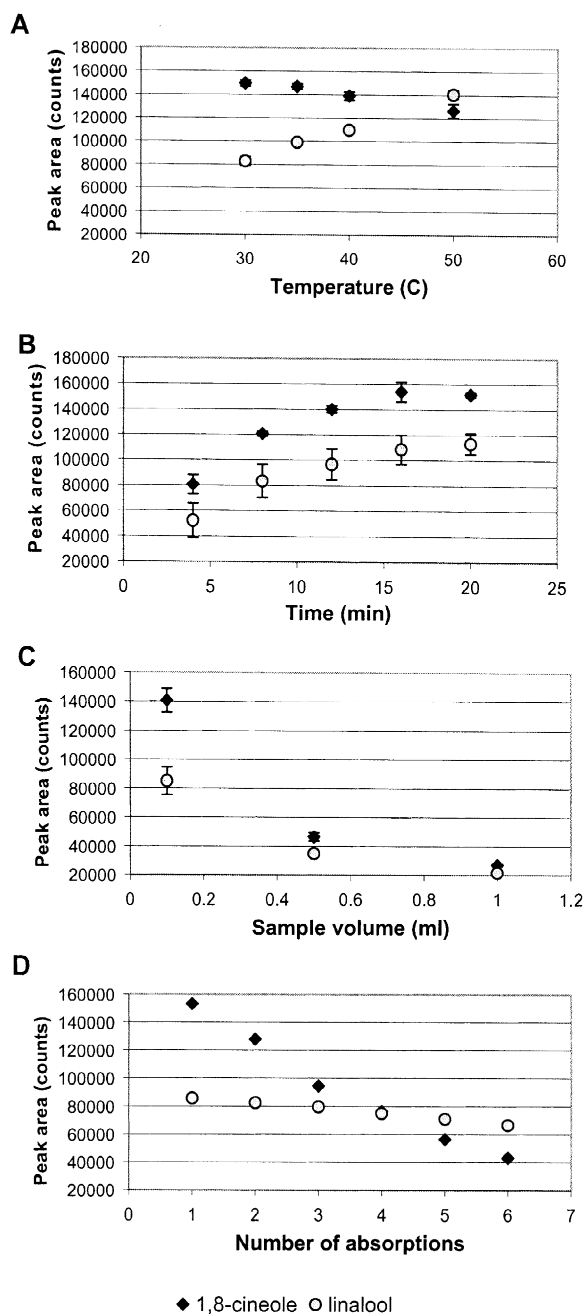

Fig. 4. Effect of SPME absorption conditions on GC peak areas of 1,8-cineole and linalool (n=3). (A) Temperature; (B) time; (C) sample volume; (D) repeated sampling of a single vial.

Table 1
Relative standard deviations (%) for within-day and between-day variability for low concentration and high concentration standards and the ratio of peak areas for 1,8-cineole and linalool from those standards ($n=5$)

	Concentration (ng/50 μl blood)	1,8-Cineole	Linalool	Ratio cineole/linalool
Within-day	25	1.6	2.3	0.9
	250	0.5	2.0	1.5
Between-day	10	4.7	8.2	7.3
	507	10.0	9.1	2.5

internal standard (22.3±4.4 vs. 4.9±1.2% per absorption, respectively).

3.3. Calibration curves

Calibration curves were linear over both concentration ranges. The curves were described by the equation $y=0.0108x+0.0054$ ($r^2=0.999$) and $y=0.0107x+0.0045$ ($r^2=0.994$) for the low and high concentration ranges, respectively. The standard errors ($n=5$) for slope and intercept for the low concentration curve were $9.70\cdot10^{-5}$ and $4.46\cdot10^{-4}$, respectively and for the high concentration curve were $3.03\cdot10^{-4}$ and $1.42\cdot10^{-2}$, respectively. The y-axis was the ratio between 1,8-cineole and linalool and the x-axis was the 1,8-cineole concentration (ng/50 μl blood).

3.4. Repeatability

The RSDs for within-day and between-day samples are shown in Table 1.

3.5. 1,8-Cineole concentration over time

Fig. 5 shows a typical chromatogram resulting from HS-SPME of blood collected after oral administration of 1,8-cineole (30 mg/kg). An example of the time profile of 1,8-cineole concentrations in blood is shown in Fig. 6.

4. Discussion

We have developed reliable quantitative assays for 1,8-cineole in expired air and whole blood using SPME. These methods will enable us to investigate the pharmacokinetics of this dietary toxin in the brushtail possum and other herbivores.

The expired air protocol evolved from the concept that breath analysis is used as an indirect measure of blood concentrations (e.g., blood alcohol measurements). Terpenes such as 1,8-cineole are volatile and therefore susceptible to being partially cleared by expiration. Sawmill workers, exposed to vaporised terpenes such as α-pinene, have been reported to clear approximately 8% of inhaled terpenes by expiration [12,14].

The protocol successfully provided a real-time indirect measure of 1,8-cineole levels in possums. Furthermore expired air analysis avoided catheterisation and the attendent use of anaesthetics, minimising stress and discomfort to animals. The results from this protocol were used to determine some preliminary pharmacokinetic parameters (t_{max}, relative C_{max} and area under the curve, AUC), however there were limitations, specifically in assay sensitivity for low concentrations. However, we did not attempt to relate expired air peaks to actual blood concentration, but developed an assay to measure blood concentrations directly.

SPME analysis of the small blood volumes available for analysis required deviation from standard HS-SPME sampling protocols that recommend a large sample volume and small headspace. Instead, our sampling strategy used a very small sample volume (final volume 100 μl) and large headspace (approximately 8.2 ml). The sample volume was insufficient to cover the bottom of the sample vial which meant stirring was not appropriate. However, because the sample's surface area to volume ratio was high, equilibration between sample and headspace readily occurred by diffusion of the analytes through the liquid phase.

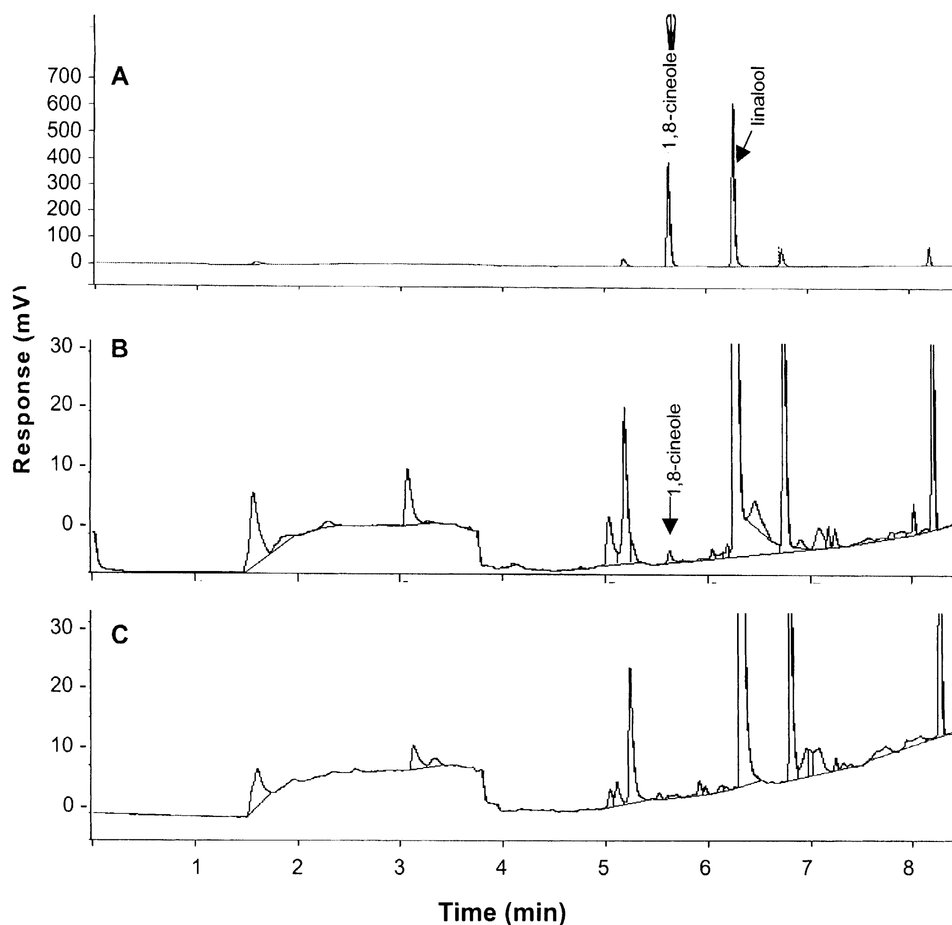

Fig. 5. Gas chromatograms (FID) from headspace SPME of (A) 50 μl blood collected 5 min after a 1,8-cineole dose (30 mg/kg), (B) low calibration standard 0.05 ng/50 μl) and (C) control blood collected prior to 1,8-cineole administration. Retention times of 1,8-cineole and linalool were 5.62 and 6.23 min, respectively.

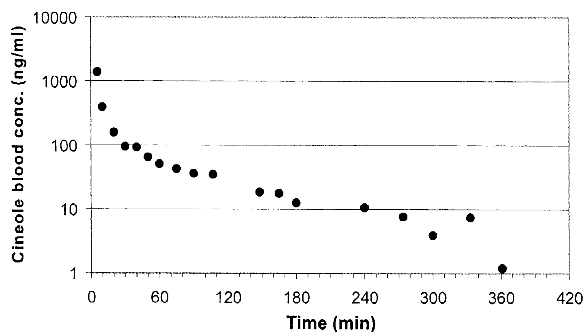

Fig. 6. Blood 1,8-cineole time profile after an oral dose of 1,8-cineole (30 mg/kg).

The choice of SPME fibre was based on the physicochemical properties of terpenes. Their volatility (1,8-cineole and linalool boiling points are 176 and 198–200 °C, respectively), lipophilicity and low molecular masses make them ideally suited for absorption onto the polydimethylsiloxane fibre. Equilibration between liquid-headspace-fibre for 1,8-cineole in this system was rapid (16 min at 35 °C).

Whole blood rather than plasma was chosen as the matrix for two main reasons. First, terpenes are lipophilic and therefore a significant proportion may be dissolved in the red blood cell fraction. Second, the volume of blood collected was too small to easily allow separation of plasma without loss of analyte

for the analyses required. Blood was collected into heparinised vials to prevent coagulation. Also there was no need to increase assay sensitivity by increasing the ionic strength or altering pH.

A 12 min equilibration time was adopted to allow SPME fibres to be loaded within the chromatography run time. Given the number of samples to be analysed for each experiment performed it was important to maximize sample through-put (four samples per hour). By 12 min, approximately 90% of equilibrium fibre loading had occurred, so there was minimal loss of sensitivity.

The optimum temperature for 1,8-cineole absorption onto the fibre was below 30 °C, and that for linalool probably exceeded 55 °C, the maximum temperature tested. An equilibrium temperature of 35 °C was chosen for the analyses as this temperature exceeded any likely ambient temperature and 1,8-cineole and linalool both maintained a high affinity for the fibre. As for sampling time, it is not necessary to use maximising conditions, so long as the temperature is constant and sufficient loading of analytes onto the fibre occurs.

Increasing the sample volume by diluting with water dramatically reduced the sensitivity of the assay. This result is not surprising as the surface area to volume ratio declines with increasing volume and, without stirring, the time required for the liquid sample and headspace to reach equilibrium increases. The addition of stirring to the protocol would probably have overcome the reduction in sensitivity at this stage. However, we decided it was not necessary to complicate the assay as the sensitivity and reliability of the assay were adequate.

In typical large sample volume–small headspace sampling systems the amount of analyte on the fibre at equilibrium is so small it can usually be neglected for semi-volatile and most volatile organic compounds [20]. The amount of analyte in the headspace can also be neglected since the headspace volume is very small compared to the sample volume [21]. However, in our small sample volume–large headspace system, the amount of analyte on the fibre and headspace is significant (Fig. 4D). Sample depletion was approximately 22 and 5% of 1,8-cineole and linalool, respectively, under our sampling conditions. Therefore in adopting the small sample volume–large headspace system it became necessary to be very accurate in measuring sample volumes in both real and calibration samples.

Linalool was chosen as the most appropriate internal standard as its chromatography was similar to 1,8-cineole. The retention times for 1,8-cineole and linalool were relatively close together (5.62 and 6.26 min, respectively) yet allowed good resolution of peaks. The other terpenes trialed as internal standards were discarded for different reasons. Limonene co-eluted with 1,8-cineole, terpinolene gave variable peak areas possibly due to its poor aqueous solubility and citronellal was avoided because of concerns over its stability.

The methods described above have become standard procedures in our laboratory. We are now using the methods as part of more extensive studies into the pharmacokinetics of 1,8-cineole in a project aiming to further understand how blood levels of dietary toxins may affect foraging behaviour in herbivores. SPME has proved to be an ideal analytical tool in this application. The technique has proven to be adaptable while maintaining excellent sensitivity and reproducibility, as well as having the obvious advantages of minimising sample preparation.

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